

TRANSMITTAL LETTER TO THE UNITED STATES
DESIGNATED/ELECTED OFFICE (DO/EO/US)
CONCERNING A FILING UNDER 35 U.S.C. 371

2727-100J

U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR 1.5)

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INTERNATIONAL APPLICATION NO
PCT/EP98/05129INTERNATIONAL FILING DATE
12 August 1998 (12.08.98)PRIORITY DATE CLAIMED
12 August 1997 (12.08.97)

TITLE OF INVENTION

"Nucleic Acid Molecule Set for Detecting Salmonella, Nucleic Acids, Kit and Use"

APPLICANT(S) FOR DO/EO/US

Cornelia Berghof, Alexander Gasch, Pia Scheu and Freimut Wilborn

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☐ This is an express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).
4. ☒ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
5. ☒ A copy of the International Application as filed (35 U.S.C. 371 (c) (2))
 - a. ☒ is transmitted herewith (required only if not transmitted by the International Bureau).
 - b. ☐ has been transmitted by the International Bureau.
 - c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☒ A translation of the International Application into English (35 U.S.C. 371(c)(2)).
7. ☒ A copy of the International Search Report (PCT/ISA/210).
8. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371 (c)(3))
 - a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
 - b. ☐ have been transmitted by the International Bureau.
 - c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
 - d. ☒ have not been made and will not be made
9. ☐ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
10. ☐ An oath or declaration of the inventor(s) (35 U.S.C. 371 (c)(4)).
11. ☒ A copy of the International Preliminary Examination Report (PCT/IPEA/409).
12. ☒ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371 (c)(5)).

Items 13 to 20 below concern document(s) or information included:

13. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
14. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
15. ☒ A **FIRST** preliminary amendment.
16. ☐ A **SECOND** or **SUBSEQUENT** preliminary amendment.
17. ☐ A substitute specification.
18. ☐ A change of power of attorney and/or address letter.
19. ☐ Certificate of Mailing by Express Mail
20. ☒ Other items or information:

declaration of the inventors (unsigned)

Sequence Listing (hard copy)

Cover page of the WIPO publication

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21. The following fees are submitted:

CALCULATIONS PTO USE ONLY

BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)) :

- ☐ Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO \$970.00
- ☒ International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO \$840.00
- ☐ International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO \$690.00
- ☐ International preliminary examination fee paid to USPTO (37 CFR 1.482) but all claims did not satisfy provisions of PCT Article 33(1)-(4) \$670.00
- ☐ International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(1)-(4) \$96.00

ENTER APPROPRIATE BASIC FEE AMOUNT =**\$840.00**

Surcharge of \$130.00 for furnishing the oath or declaration later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492 (e)).

\$0.00

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE
Total claims	21 - 20 =	1	x \$18.00
Independent claims	3 - 3 =	0	x \$78.00

\$18.00**\$0.00**Multiple Dependent Claims (check if applicable). ☐**\$0.00****TOTAL OF ABOVE CALCULATIONS =****\$858.00**

Reduction of 1/2 for filing by small entity, if applicable. Verified Small Entity Statement must also be filed (Note 37 CFR 1.9, 1.27, 1.28) (check if applicable). ☐

\$0.00**SUBTOTAL =****\$858.00**

Processing fee of \$130.00 for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492 (f)).

\$0.00**TOTAL NATIONAL FEE =****\$858.00**

Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31) (check if applicable). ☐

\$0.00**TOTAL FEES ENCLOSED =****\$858.00**Amount to be:
refunded

\$

charged

\$

☐ A check in the amount of _____ to cover the above fees is enclosed.

☒ Please charge my Deposit Account No. **501145** in the amount of **\$858.00** to cover the above fees.
A duplicate copy of this sheet is enclosed. (**Order No. 2727-100J**)

☒ The Commissioner is hereby authorized to charge any fees which may be required, or credit any overpayment to Deposit Account No. **501145**. A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

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REGISTRATION NUMBER

February 9, 2000

DATE

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2727-100J

IN THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US)

Applicants: Cornelia Berghof, Alexander Gasch,
Pia Scheu and Freimut Wilborn

Int. Appln No.: PCT/EP98/05129

Int. Filing Date: 12 August 1998 (12.08.98)

Priority Date Claimed: 12 August 1997 (12.08.97)

For: NUCLEIC ACID MOLECULE SET FOR DETECTING
SALMONELLA, NUCLEIC ACIDS, KIT AND USE

PRELIMINARY AMENDMENT

Box PCT
Commissioner for Patents
Washington, D.C. 20231
Attn: DO/EO/US

S I R:

Preliminary to examination of the above-identified
application kindly amend the application as follows:

In the Claims:

In claim 4, line 1, kindly delete "2 or 3,";

In claim 5, line 1, kindly delete "any one of the preceding
claims" and substitute therefor --claim 1--;

In claim 6, line 1, kindly delete "any one of the preceding
claims" and substitute therefor --claim 1--.

In claim 7, line 2, kindly delete "any one of the preceding
claims" and substitute therefor --claim 1--;

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In claim 7, lines 7-8, kindly delete "any one of claims 1, 2 or 3" and substitute therefor --claim 1--;

In claim 9, lines 3, 6, 8 and 11, kindly delete each occurrence of "either" and "or claim 8";

In claim 10, lines 1-2, kindly delete "any one of claims 7 to 9" and substitute therefor --claim 7--;

In claim 11, lines 1-2, kindly delete "any one of claims 7 to 10" and substitute therefor --claim 7--;

In claim 12, lines 1-2, kindly delete "any one of claims 7 to 11" and substitute therefor --claim 7--;

In claim 14, lines 1-2, kindly delete "or claim 13";

In claim 15, lines 4-5, kindly delete "any one of claims 1 to 6" and substitute therefor --claim 1--;

In claim 15, lines 6-7, kindly delete "any one of claims 7 to 14" and substitute therefor --claim 7--;

In claim 18, lines 1-2, kindly delete "any one of claims 1 to 6" and substitute therefor --claim 1--;

In claim 18, line 3, kindly delete "any one of claims 7 to 14" and substitute therefor --claim 7--;

In claim 18, line 4, kindly delete "any one of claims 15 to 17" and substitute therefor --claim 15--;

In claim 18, line 7, kindly delete ", 2 or 3";

In claim 21, line 1, kindly delete " 19 or 20,";

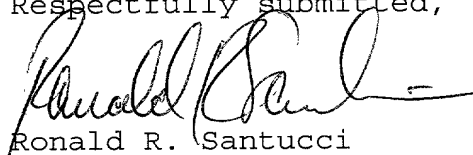
In claim 21, line 6, kindly delete "any one of claims 7 to 14" and substitute therefor --claim 7--;

In claim 21, line 9, kindly delete ", 2 or 3".

REMARKS

The claims of the above-identified application (the claims as amended under Article 34 of the PCT) have been amended to remove all multiple dependencies. No new matter has been added. Accordingly, an early examination of the application is respectfully requested.

Respectfully submitted,



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Nucleic acid molecule set for detecting *Salmonella*, nucleic acids, kit and use

In the foodstuffs and pharmaceutical industries the contamination of products by pathogenic microorganisms by way of the raw materials used or during the production or packaging processes poses a major problem. *Salmonellae* are among the most serious pathogens transmitted to humans through foodstuffs. Since the detection and identification of *Salmonellae* by conventional microbiological detection processes is very time-consuming - at least five days are required for the increase in quantity and subsequent serotyping required by legal regulations (LMBG, FDA) - there is a great need for alternative rapid methods.

In recent years, a number of new methods have been developed for routine use to detect microorganisms. These include immunological processes based on the use of polyvalent or monoclonal antibodies and processes in which nucleic acid probes are used for detection by means of hybridisation to organism-specific nucleic acids. Further methods that have been described are those processes based on a specific nucleic acid amplification, with or without a subsequent confirmation reaction by nucleic acid hybridisation. Suitable processes for the amplification of nucleic acids are, for example, polymerase chain reaction [PCR] [US Patents 4,683,195; 4,683,202; and 4,965,188], ligase chain reaction [WO Publication 89/09835], "self-sustained sequence replication" [EP 329 822], the "transcription based amplification system" [EP 310 229] and the Q β RNA-replicase system [US Patent 4,957,858].

The mentioned nucleic-acid-based processes are so sensitive that, unlike conventional microbiological processes, a lengthy increase in quantity of the microorganism to be detected from the sample to be investigated is unnecessary. An investigation of the presence or absence of, for example, *Salmonellae* is therefore generally concluded within one working day when using the mentioned nucleic-acid-based processes.

Some nucleic acid sequences for detecting *Salmonellae* by polymerase chain reaction are known. A disadvantage is, however, that when using those nucleic acid sequences as primers in the polymerase chain reaction false positive results [WO 95/33854] or false negative results [WO 92/01056; WO 95/00664; WO 92/01056; WO 93/04202] occur. In other cases, only an insufficient number of strains of all 7 *Salmonellae* subspecies have been studied [WO 92/08805; WO 94/25597; DE 4337295], so that thus far it is unclear whether the nucleic acid sequences in question are suitable for detecting all *Salmonella* strains.

An advantage of, for example, the primers and probes described in International Patent Application WO 95/00664 is that they allow the highly selective detection of bacteria of the *Salmonella* genus without the occurrence of false positive results. A disadvantage when using the oligonucleotides according to WO 95/00664 in amplification processes such as polymerase chain reaction is, however, the fact that none of the described primer pairs enable detection of all the representatives of the 7 *Salmonella* subspecies. For example, when using the primers ST11/ST15,

a number of representatives of subspecies IIIa (subsp. *arizonae*) are not detected, and when using the primers ST11/ST14 a number of representatives of subspecies I (subsp. *enterica* Serovar. Blockley) and of subspecies IIIa (subsp. *arizonae*) are not detected.

An aim of the invention described herein was to optimise the detection processes described in WO 95/00664 by finding nucleic acid sequences the use of which as primers and/or probes ensures as complete detection as possible of all the representatives of the *Salmonella* genus.

According to an embodiment, the problem underlying the invention is solved by a set of nucleic acid molecules by means of which, in a process for the detection of representatives of *Salmonella enterica* subsp. *enterica*, *salamae*, *arizonae*, *diarizonae*, *houtenae*, *bongori* and *indica*, all the representatives of those subspecies can be detected, the set being obtainable by

- (a) obtaining or deriving a first nucleic acid molecule (nucleic acid molecule 1) in a manner known *per se* using a nucleic acid isolate of a representative of one of the mentioned *Salmonella enterica* subspecies, which first nucleic acid molecule is specifically suitable as primer or probe for the detection of that representative or of further or all representatives of that one *Salmonella enterica* subspecies and possibly also of representatives of further *Salmonella enterica* subspecies,
- (b) obtaining or deriving a second nucleic acid molecule (nucleic acid molecule 2) in a manner known *per se* using a nucleic acid isolate of a different representative of one of the mentioned *Salmonella enterica* subspecies, which

second nucleic acid molecule is specifically suitable as primer or probe for the detection of that representative or of further or all representatives of that different *Salmonella enterica* subspecies and possibly also of representatives of others of the mentioned *Salmonella enterica* subspecies, and

(c) unless it is already possible to detect all the representatives of the mentioned *Salmonella enterica* subspecies using the nucleic acid molecules obtainable according to (a) and (b), continuing to obtain or derive nucleic acid molecules according to (a) and/or (b) until all the representatives of the mentioned *Salmonella enterica* subspecies can be detected using the obtained or derived set of nucleic acid molecules.

A derived nucleic acid molecule may be a nucleic acid molecule that can be hybridised with the obtained nucleic acid molecule and that preferably has the same number of bases, possible hybridisation conditions being:

temperature $\geq 25^{\circ}\text{C}$ and 1M NaCl concentration.

A derived nucleic acid molecule may be, for example, a nucleic acid molecule the sequence of which has been determined by computer design and that has subsequently been manufactured and obtained by chemical synthesis.

The solution to the problem underlying the invention can also be described as the provision of one or more nucleic acid molecule(s) Y (Z, ...), that(those) nucleic acid molecule(s) being characterised in that the use of that(those) nucleic acid molecule(s) - in addition to the use of a nucleic acid molecule (X) - in a process for the

detection of bacteria of the *Salmonella* genus enables the detection also of *Salmonella* strains or *Salmonella* isolates that cannot be detected or can be detected only with relatively low sensitivity using the nucleic acid molecule (X).

The set of nucleic acid molecules according to the invention can be characterised in that the nucleic acid isolates comprise or are phylogenetically conserved base sequences or regions of those base sequences. For the term "phylogenetically conserved base sequence", see, for example, WO 95/00664 or Herder's Lexikon der Biochemie und Molekularbiologie, supplemented 1995, page 132, spectrum, production, etc..

The set of nucleic acid molecules according to the invention can be characterised in that the individual nucleic acid molecules or some of the nucleic acid molecules hybridise to

- (i) different phylogenetically conserved base sequences, or
- (ii) one and the same phylogenetically conserved base sequence at non-overlapping sequence regions, or
- (iii) one and the same phylogenetically conserved base sequence at overlapping sequence regions.

The set of nucleic acid molecules according to the invention or a set of nucleic acid molecules according to the invention by means of which, in a process for the detection of representatives of *Salmonella enterica* subsp. *enterica*, *salamae*, *arizonae*, *diarizonae*, *houtenae*, *bongori* and *indica*, all the representatives of those subspecies can

be detected, can be characterised in that the set for an individual nucleic acid molecule, for a number of its individual nucleic acid molecules or for each of its individual nucleic acid molecules in each case comprises at least one further nucleic acid molecule that, in a region of at least 10 successive nucleotides of their nucleotide chain, corresponds to less than 100% but to at least 80% of the base sequence.

Such a set of nucleic acid molecules according to the invention can be characterised in that the set for an individual nucleic acid molecule, for a number of its individual nucleic acid molecules or for each of its individual nucleic acid molecules in each case comprises at least one further nucleic acid molecule that, in a region of at least 10 successive nucleotides of their nucleotide chain, differs from the other or further nucleic acid molecule in precisely one base position.

A set of nucleic acid molecules according to the invention can be characterised in that it comprises one or more, but not exclusively, nucleic acid molecules that are fragments of the SEQ ID NO 1 according to WO 95/00664 or of its complementary sequence.

A set of nucleic acid molecules according to the invention can also be characterised in that the individual nucleic acid molecules hybridise to the same strand of nucleic acid isolates of representatives of *Salmonella enterica* subspecies that are being subjected to the process for their detection.

The problem underlying the invention is also solved by a nucleic acid molecule that belongs to a set of nucleic acid molecules according to the invention or that can be used for such a set, the nucleic acid molecule being characterised in that, in a region of at least 10 successive nucleotides of its nucleotide chain, the sequence of the nucleic acid molecule corresponds exactly to a sequence region of at least one representative of the mentioned *Salmonella enterica* subspecies, the sequence region comprising or being a phylogenetically conserved base sequence or a region of that base sequence.

Such a nucleic acid molecule according to the invention can be characterised in that, in a region of at least 10 successive nucleotides of its nucleotide chain, it is 100% or at least 80% identical to a corresponding number of successive nucleotides of one or more of the following sequences or their complementary sequences:

SEQ ID NO: 1	ATGGATCAGAATACGCCCCG
SEQ ID NO: 2	ATGGATCAGAATACACCCCCG
SEQ ID NO: 3	CAGAATACGCCCCGTTTCGGC
SEQ ID NO: 4	CAGAATACACCCCCGTTTCGGC
SEQ ID NO: 5	CAGAATACGCCCCGTTTCAGC
SEQ ID NO: 6	CAACCTAACTTCTGCGCCAG
SEQ ID NO: 7	CAACCTAACTTCTGCACCAG
SEQ ID NO: 8	CAACCTAACCTCTGCGCCAG
SEQ ID NO: 9	CAACCTAACTTCTGCGCCAG

The problem underlying the invention is also solved by a nucleic acid molecule characterised in that, in respect of its sequence, it is homologous to an above-characterised

nucleic acid molecule according to the invention and, in at least 10 successive nucleotides of its nucleotide chain,

- (i) is identical to an above-characterised nucleic acid molecule according to the invention, or
- (ii) differs from an above-characterised nucleic acid molecule according to the invention in not more than one nucleotide, or
- (iii) differs from an above-characterised nucleic acid molecule according to the invention in not more than two nucleotides.

A nucleic acid molecule according to the invention can be characterised in that it is from 10 to 250 nucleotides long and preferably from 15 to 30 nucleotides long.

A nucleic acid molecule according to the invention can also be characterised in that it is single-stranded or has a complementary strand.

A nucleic acid molecule according to the invention can also be characterised in that it is present

- (i) as DNA, or
- (ii) as RNA corresponding to (i), or
- (iii) as PNA, the nucleic acid molecule where appropriate having been modified or labelled in a manner known *per se* for analytical detection processes, especially detection processes based on hybridisation and/or amplification.

A nucleic acid molecule according to the invention can also be characterised in that it is a modified or labelled nucleic acid molecule in which up to 20% of the nucleotides

of at least 10 successive nucleotides of its nucleotide chain are building blocks known *per se* as probes and/or primers, especially nucleotides that do not occur naturally in bacteria.

A nucleic acid molecule according to the invention can also be characterised in that it is a modified or labelled or additionally modified or labelled nucleic acid molecule that comprises, in a manner known *per se* for analytical detection processes, one or more radioactive groups, coloured groups, fluorescent groups, groups for immobilisation on a solid phase, groups for an indirect or direct reaction, especially for an enzymatic reaction, preferably using antibodies, antigens, enzymes and/or substances having an affinity for enzymes or enzyme complexes, and/or other modifying or modified groups of nucleic-acid-like structure that are known *per se*.

The problem underlying the invention is also solved by a kit for analytical detection processes, especially for the detection of bacteria of the *Salmonella* genus, that kit being characterised by

- (i) a set of nucleic acid molecules according to the invention, or
- (ii) one or more nucleic acid molecules according to the invention.

A kit according to the invention can thus comprise a set of nucleic acid molecules according to the invention or one or more nucleic acid molecules according to the invention, there additionally being provided the other customary components for nucleic acid hybridisations or nucleic acid

amplifications, for example a polymerase, a reverse transcriptase, a ligase or an RNA-polymerase, see, for example, WO 95/00664.

A set of nucleic acid molecules of the kit according to the invention will preferably be produced synthetically in at least two separate synthesis batches. The kit according to the invention preferably does not comprise any degenerate nucleic acid molecules.

Finally, the problem underlying the invention is solved by the use of a set of nucleic acid molecules according to the invention or of a kit according to the invention to detect the presence or absence of bacteria belonging to a group of bacteria of the *Salmonella* genus, especially of representatives of the above-mentioned *Salmonella enterica* sub-species.

For the use according to the invention, nucleic acid hybridisation and/or nucleic acid amplification can be carried out.

As nucleic acid amplification, there can be carried out a polymerase chain reaction (PCR).

For the use according to the invention, differences between the genomic DNA and/or RNA of the bacteria to be detected and of the bacteria that are not to be detected can be determined at at least one nucleotide position in the region of a nucleic acid molecule according to the invention and representatives of a group of bacteria of the

Salmonella genus can be detected, especially representatives of the mentioned *Salmonella enterica* subspecies.

To detect *Salmonellae* by means of nucleic acid hybridisation or amplification, *Salmonella*-specific oligonucleotides are used. *Salmonella*-specific oligonucleotides are nucleic acid molecules, from 10 to 250 bases (preferably from 15 to 30 bases) long, the base sequence of which is characteristic for *Salmonellae*: when using such oligonucleotides as primers or probes - with suitable reaction conditions - hybridisation/amplification takes place only when DNA of the *Salmonellae* to be detected is present in the test sample, but not when DNA of other bacteria is present.

As described below, given certain prerequisites non-specific oligonucleotides may also be used as primers or probes. Such oligonucleotides enable hybridisation and/or amplification not only when *Salmonella*-DNA is present in the sample but also in the presence of DNA of a bacterium or of a number of bacteria not belonging to the *Salmonella* genus.

Since, in highly conserved gene regions, substitutions (e.g. point mutations) in the DNA can occur even in the case of very closely related bacteria, comprehensive DNA sequencing or specificity tests (e.g. by carrying out PCR) are necessary to select suitable oligonucleotides. This applies equally to bacteria to be detected (i.e. *Salmonellae*) and to bacteria that are not to be detected (i.e. bacteria not belonging to the *Salmonella* genus).

To detect *Salmonellae*, firstly nucleic acids, preferably genomic DNA, are released from the cells contained in a sample or bacterial culture to be investigated. By means of nucleic acid hybridisation, the direct detection of *Salmonella* nucleic acids in the sample to be investigated can then be effected using the *Salmonella*-specific oligonucleotides according to the invention as probe. Various processes known to the person skilled in the art are suitable for that purpose, such as, for example, "Southern blot" or "dot blot".

Preference is given, however, above all on account of the relatively high sensitivity, to indirect detection in which the DNA/RNA sequences sought are firstly amplified by means of the above-mentioned processes for amplifying nucleic acids, preferably PCR. The amplification of DNA/RNA is effected by using *Salmonella*-specific oligonucleotides. In that process specific amplification products are formed only when *Salmonella*-DNA/RNA is present in the sample to be investigated. The specificity of the detection process can be increased by a subsequent detection reaction using *Salmonella*-specific oligonucleotides as probes. It is also possible to use non-specific oligonucleotides as probes.

Alternatively, amplification can also be carried out in the presence of one or more non-specific oligonucleotides, so that possibly also DNA/RNA of other microorganisms that are not to be detected may be amplified. Such an amplification process is generally less specific and should therefore be backed up by a subsequent detection reaction using *Salmonella*-specific oligonucleotides as probe.

Various processes by which amplification products formed in the indirect processes can be detected are known to the person skilled in the art. These include, *inter alia*, visualisation by means of gel electrophoresis, the hybridisation of probes on immobilised reaction products [coupled to nylon or nitrocellulose filters ("Southern blots") or, for example, on beads or microtitre plates] and the hybridisation of the reaction products on immobilised probes (e.g. "reverse dot blots" or beads or microtitre plates coupled with probes).

A large number of different variants have been described by means of which the described *Salmonella*-specific or non-specific oligonucleotides for use as probes and/or primers in direct or indirect detection processes can be labelled or modified. They may comprise, for example, radioactive, coloured or fluorescent groups or groups that enable immobilisation on a solid phase or groups that have been modified or that modify in some other way, such as, for example, antibodies, antigens, enzymes or other substances having an affinity for enzymes or enzyme complexes. Probes and primers may be either naturally occurring or synthetically produced double- or single-stranded DNA or RNA or modified forms of DNA or RNA, such as, for example, PNA (in those molecules the sugar units have been replaced by amino acids or peptides). Individual nucleotides or a number of nucleotides of the probes or primers may be replaced by analogous building blocks (such as, for example, nucleotides that do not naturally occur in the target nucleic acid). In the case of the above-mentioned indirect detection processes, the detection can be carried out also by means of an internally labelled amplification

product. That can be effected, for example, by the integration of modified nucleoside triphosphates (e.g. coupled with digoxigenin or fluorescein) during the amplification reaction.

Suitable *Salmonella*-specific oligonucleotides according to the invention are nucleic acids, preferably from 15 to 30 bases long, that correspond, at least in a 10 base long sequence, to sequences 1 to 10 or to their complementary sequences. Relatively small differences (1 or 2 bases) in that 10 base long sequence are possible without loss of the requisite specificity in the amplification and/or hybridisation. The person skilled in the art will know that in the case of such relatively small differences the reaction conditions need to be altered accordingly.

In order to enable complete detection of all the *Salmonella* strains using the DNA region outlined in WO 95/00664, comprehensive DNA sequence analyses were necessary. The sequence of that DNA region was determined from 37 selected *Salmonella* strains of all 7 subspecies (for most of the strains, the sequence of the DNA region between primers ST15 and ST11; this corresponds to position 1275 to 1654 of SEQ ID NO: 1 in WO 95/00664). Possible experimental procedures will be known to the person skilled in the art and will not be described here in detail; a brief summary of the results will be given. DNA of the selected *Salmonella* strains was prepared by standard procedures and the relevant region was amplified by PCR and subsequently sequenced. In the PCR and the subsequent sequencing, the following primers were used for most of the *Salmonella* strains:

ST11: AGCCAACCATTTGCTAAATTGGCGCA (see claim 3,
WO 95/00664)
ST15: GGTAGAAATTCCCAGCGGGTACTG (see claim 3,
WO 95/00664).

Since, however, no amplification, or only insufficient amplification, occurred with that primer pair with a number of strains of subspecies IIIa, IV, V and VI, in those cases the following primers were used for the PCR and sequencing:

ST11: AGCCAACCATTTGCTAAATTGGCGCA (see claim 3,
WO 95/00664)
ST14: TTTGCGACTATCAGGTTACCGTGG (see claim 3,
WO 95/00664).

A comparison of the DNA sequences of all 37 *Salmonella* strains showed that while it was as a whole a conserved DNA region, the degree of conservation appeared at first glance to have only limited suitability for deriving *Salmonella*-specific oligonucleotides. Even in the most highly conserved regions, base substitutions were observed in some of the sequenced strains. Interestingly, it was found that many of the base substitutions occur only within a subgroup and that the substitutions are moreover generally conserved within that subgroup. This suggested the possibility of using more than two primers in the PCR in order to enable amplification also of those variants in which one or more base substitutions are present in the region of the primer binding sites. As the person skilled in the art will know, for that purpose there are customarily used

degenerate primers or primers having deoxyinosin at the variable sites. A number of degenerate oligonucleotides that were potentially suitable as primers for the detection of all *Salmonella enterica* subspecies were therefore deduced from the above-mentioned sequence comparison. It was found, however, that those degenerate primers have only limited suitability for PCR detection since they result in an increase in the occurrence of non-specific reaction products, especially in the case of sequence regions of high complexity. Since the sensitivity of the PCR detection generally suffers from the occurrence of such non-specific reaction products, a different procedure was tried. "Complementing" primers were used in the PCR. In contrast to degenerate primers, in which all the possible combinations of the individual base substitutions are represented in the primer mixture (number of primers = $2^x \times 3^y \times 4^z$ where x, y and z are the number of positions at which two, three or four different bases are observed in the region of the primer binding site), in such complementing primers only the actually occurring sequences are present. The advantage over degenerate primers lies in the lesser complexity of the primer mixture according to the invention, as a result of which the probability that non-specific amplification products will be formed is markedly reduced. As has been shown in a number of experiments, this is especially advantageous in PCR detection using samples having a high content of "non-specific" DNA (DNA that does not come from bacteria to be detected) since, otherwise, the sensitivity of the detection may be radically reduced.

A major advantage when using complementing oligo-nucleotides/primers lies in the possibility of optimising existing detection processes. For example, it is possible that individual false negative results can be eliminated by additionally using in the PCR and/or hybridisation reaction oligonucleotides comprising the sequence of the previously undetected strains.

The DNA sequence comparison yielded a number of relatively short DNA regions that appeared to be potentially suitable for the strategy described (use of in total ≥ 3 primers in the PCR) for optimising the *Salmonella* detection process. The following Example is given by way of clarification.

Example 1: Detection of *Salmonella* strains of all 7 subspecies by polymerase chain reaction

The following 3 sections of the sequenced DNA region are to serve as examples for the sequence variations observed:

Section I (position 1336 to 1355 of SEQ ID NO: 1 in WO 95/00664)

SEQ ID NO: 1	ATGGATCAGAATACGCCCCG
SEQ ID NO: 2	ATGGATCAGAATACACCCCG

Section II (position 1342 to 1361 of SEQ ID NO: 1 in WO 95/00664)

SEQ ID NO: 3	CAGAATACGCCCCGTTTCGGC
SEQ ID NO: 4	CAGAATACACCCCGTTTCGGC
SEQ ID NO: 5	CAGAATACGCCCCGTTTCAGC

Section III (complementary to position 1483 to 1502 of
SEQ ID NO: 1 in WO 95/00664)

SEQ ID NO: 6	CAACCTAACTTCTGCGCCAG
SEQ ID NO: 7	CAACCTAACTTCTGCACCAG
SEQ ID NO: 8	CAACCTAACCTCTGCGCCAG
SEQ ID NO: 9	CAACCTAACTTCTGCGGCAG
SEQ ID NO: 10	CAGCCTAACTTCTGCGCCAG

To test whether those sequence sections are suitable for
detecting all the *Salmonella* strains of the 7 subspecies,
the oligonucleotides Sa 1 to 10 were used in the PCR in the
following combinations:

Primer combination 1:

Sa1/Sa2	(each in a final concentration of 0.2 μ M)
Sa6/Sa7/Sa8/Sa9/Sa10	(each in a final concentration of 0.08 μ M)

Primer combination 2:

Sa3/Sa4/Sa5	(each in a final concentration of 0.13 μ M)
Sa6/Sa7/Sa8/Sa9/Sa10	(each in a final concentration of 0.08 μ M)

DNA was isolated by standard processes from pure cultures
of the *Salmonella* strains listed in Table 1a.

Approximately from 10 to 100 ng of each of those DNA
preparations was then used in the PCR in the presence of
primer combination 1 or primer combination 2, 200 μ M of
dNTP's (Boehringer Mannheim), 1.5 mM MgCl₂, 16 mM (NH₄)₂SO₄,

67 mM Tris/HCl (pH 8.8), 0.01% Tween 20 and 0.03 U/ μ l Taq-polymerase (Biomaster). The PCR was carried out in a Perkin-Elmer 9600 thermocycler having the following thermoprofile:

- initial denaturing	95°C	5 min
- amplification (35 cycles)	95°C	30 sec
	63°C	90 sec
- final synthesis	72°C	5 min

After the end of the PCR reaction, the amplification products were separated by means of agarose gel electrophoresis and visualised by staining with ethidium bromide. The expected product of 167 bp length (primer combination 1) or of 161 bp length (primer combination 2) was observed in all cases in which DNA of strains of the *Salmonella* genus was present (compare Table 1a), but not in the presence of DNA of other tested bacteria (compare Table 1b). After the end of the run, the DNA contained in the gels was transferred by standard methods to nylon filters and hybridised with the oligonucleotide ST14 (TTTGCGACTATCAGGTTACCGTGG (see claim 3, WO 95/00664)) labelled at the 5' end with digoxigenin to test the base specificity especially sensitively. Hybridisation was effected in 5 x SSC, 2% blocking reagent, 0.1% lauryl sarcosine, 0.02% SDS and 5 pmol/ml of probe for 4 hours at 60°C. Washing was carried out in 2 x SSC, 0.1% SDS for 2 x 15 minutes at 60°C. Detection was carried out according to standard methods using anti-digoxigenin/alkaline

phosphatase conjugates in the presence of 5-bromo-4-chloro-3-indolyl phosphate and 4-nitro-blue tetrazolium chloride (Boehringer Mannheim).

A band was observed on the filters only in those cases in which a band had previously been visible on the agarose gel (see Table 1a). Thus, the presence of the 296 tested *Salmonella* strains from each of the 7 subspecies was detected both by PCR and by hybridisation. A positive signal was obtained for each of those strains with primer combination 1, primer combination 2 and, in the subsequent confirmation reaction, by hybridisation with the probe ST14. By contrast, none of the tested bacterial strains not belonging to that genus was detected using this system.

Table 1a: Positive *Salmonella* strains in PCR amplification using the two primer combinations 1 or 2 and in the subsequent hybridisation using the oligonucleotide ST14.

No.	Subspecies	Serogroup	Serovar.
	<i>S. enterica</i> subsp. <i>Enterica</i>	B	Abony
			Abortusovis
			Africana
			Agona
			Agona, lactose +
			Arechavaleta
			Brandenburg
			Bredeney
			Chester
			Coeln
			Derby O: 5 -
			Duisburg
			Duisburg, monophasic
			Heidelberg
			Heidelberg, O5-
			I 4, 12: d: -
			I 4, 12:-:-
			I 9, 12: l, v: -
			Indiana
			Kiambu
			Kunduchi
			Paratyphi B
			Paratyphi B H 1,2 negative
			Paratyphi B O5 -

			Reading
			Saintpaul 05 -
			Saintpaul
			Sandiego
			Schleisheim
			Schwarzengrund
			Stanley
			Stanleyville
			Typhimurium
			Typhimurium 4 : i : 1,2 (0:5-)
			Typhimurium 0 : 5 -
			Typhimurium, TA 1535
			Typhimurium, TA 1537
			Typhimurium, TA 1538
			Typhimurium, TA 97
			Typhimurium, TA 98
			Typhimurium, TA 100
		C ₁	Augustenborg
			Bareilly
			Braenderup
			Choleraesuis
			Choleraesuis var. Decatur
			Choleraesuis var. Kunzendorf
			Colindale
			Concord
			Infantis
			Isangi
			Lille
			Livingstone
			Mbandaka

004740 "4E45460

			Mikawasima
			Montevideo
			Ohio
			Oranienburg
			Oslo
			Richmond (2 isolates tested)
			Rissen
			Singapore
			Tennessee
			Thompson (2 isolates tested)
			Virchow
			I 6, 7 : - : - (2 isolates tested)
		C ₂ C ₃	Albany (2 isolates tested)
			Altona
			Apeyeme
			Bardo
			Blockley
			Bovismorbificans
			Charlottenburg
			Cottbus
			Emek
			Ferruch
			Glostrup
			Goldcoast
			Haardt
			Hadar
			Kentucky
			Litchfield

			Manchester
			Manhattan
			Molade
			München
			Newport
			Takoradi
			I 6, 8 : - : -
			I 8, 20 : - : -
		D ₁	Dublin
			Durban
			Enteritidis
			Enteritidis plasmid phage 37MD
			Enteritidis PT 4/6
			Enteritidis, phage
			Gallinarum
			Gallinarum-Pullorum
			Israel
			Javiana
			Kapemba
			Napoli
			Panama
			Pullorum
			I 9, 12 : - : -
		D ₂	Plymoth
		E ₁	Amager
			Amsterdam O : -, 15+, 34+
			Anatum
			Anatum O 15+
			Anatum O : 10 -, O : 15+
			Birmingham

00110 "4E45460

			Butantan
			Falkensee
			Give
			Lexington
			London
			Meleagridis
			Münster
			Münster, O : 10 -, 15+
			Orion
			Orion O : 10 -, 15+ 34+
			Sinstorf
			Stockholm
			Uganda (2 isolates tested)
			Vejle (2 isolates tested)
			Weltevreden
			Westhampton
			Zanzibar
			I 3, 10 : - : 6 (monophase)
			I 10 : - : 1,6
		E ₄	Abaetuba
			Aberdeen
			Cannstatt
			Llandoff
			Senftenberg, delayed Lac. +
			I 1, 3, 19, : - : -
		F	Chandans (2 isolates tested)
			Kisarawe
			Krefeld
			Liverpool
			Rubislaw

004404458460

			Senftenberg
			Solt
			Telashomer
		G	Grumpensis
			Havana
			Idikan
			Kedougou
			Poona
			Putten
			Worthington
			I 13, 23, : -
		H	Caracas
			Charity
			Lindern
			Onderstepoort
			Sundsvall
		I	Gaminara
			Hvittingfoss
			Malstatt
			Saphra
		J	Bonames
		K	Cerro
		L	Minnesota (2 isolates tested)
			Ruiru
		M	Cotham
			Guildford
			Ilala
			Loeben
			Mundonobo
			Nima

			Patience
			Pomona
			Taunton
			Wedding
		N	Aqua
			Morningside
			Urbana
		O	Adelaide
			Alachua
			Ealing
			Haga
			Monschaui
		P	Lansing
			Roan (2 isolates tested)
			Shettfield
		Q	Kokomelemle
		R	Johannesburg
		S	Waycross (2 isolates tested)
		T	Waral
		U	Thetford
		V	Koketime (2 isolates tested)
			Lawra
		W	Suelldorf
		X	I 47, z ₄ , z ₂₃ : - (monophase)
			Mountpleasant
	<i>S. enterica</i> subsp. <i>salamae</i>	B	II 4, 12 : a : -

		C ₁	II 6, 7 : d : 1,7
		F	II 11 : g, m, s, t: z ₃₉
		I	II 16 : g, m, s, t: -
		J	II 17 : c : z ₃₉
			II 17 : b : e, n, x, z ₁₅
		L	II 21 : z ₁₀ : -
		P	II 38 : d : 1,5
		R	II 1, 40 : z ₄₂ : 1,5,7
		S	II 41 : z ₁₀ , 1, 2
		T	II 42 : r : - (3 isolates tested)
		X	II 47 : a : 1,5 (2 isolates tested)
			II 47 : b : 1,5 (2 isolates tested)
			II 47 : b : z ₆
		Z	II 50 : b : z ₆ (5 isolates tested)
		O : 58	II 58 : 1, z ₁₃ , z ₂₈ : z ₆
	<i>S. enterica</i> subsp. <i>arizonae</i>	J	IIIa 17 : z ₄ , z ₃₂ : -
		K	IIIa 18 : z ₄ , z ₂₃ : -
		P	IIIa 38 : l, v: -
		R	IIIa 40 : z ₄ , z ₂₄ : -
		S	IIIa 41: z ₄ , z ₂₃ : -
		U	IIIa 43 : g, z ₅₁ : -
		V	IIIa 44 : z ₄ , z ₃₂ : -
			IIIa 44 : z ₄₁ , z ₂₃ : -
		Y	IIIa 48 : (l) : -
			IIIa 48 : g, z ₅₁ : -

			IIIa 48 : z ₃₆ : -
			IIIa 48 : z ₄ , z ₂₃ : -
		Z	IIIa 50 : z ₄ , z ₂₄ : -
		O : 51	IIIa 51 : z ₄ , z ₂₃ : -
			IIIa 51 : g, z ₅₁ : -
		O : 53	IIIa 53 : z ₄ , z ₂₃ , z ₃₂ : -
			IIIa 53 : z ₂₉ : -
		O : 62	IIIa 62 : z ₃₆ : -
		O : 63	IIIa 63 : g, z ₅₁ : -
	<i>S. enterica</i> subsp. <i>diarizonae</i>	D ₁	IIIb 1, 9, 12 : y : z ₃₉
		I	IIIb 16 : k : -
		J	IIIb 17 : z ₁₀ , e, n, x, z ₁₅
		O	IIIb 35 : k: e, n, z ₁₅
		P	IIIb 38 : l, v: z ₅₃
			IIIb 38 : l, v: z ₅₄
		T	IIIb 42 : k : z ₃₅
		X	IIIb 47 : b : z ₆
			IIIb 47 : k : z ₃₅
			IIIb 47 : r: z ₅₃
			IIIb 47 : - : -
		Y	IIIb 48 : (k) : z ₅₃
		Z	IIIb 50 : k : z
			IIIb 50 : r : z
		O : 53	IIIb 53 : l, k : z
		O : 60	IIIb 60 : z ₅₂ : z ₅₃
		O : 61	IIIb 61 : i : z
			IIIb 61 : l, v : 1,5,7
			IIIb 61 : l, v : 1,5,7: (z ₅₇)

			IIIB 61 : r : z ₅₃
	<i>S. enterica</i> subsp. <i>houtenae</i>	F	IV 11 : z ₄ , z ₂₃ : -
		I	IV 16 : z ₄ , z ₃₂ : -
		I	IV 16 : z ₄ , z ₃₂ : -
		J	IV 17 : z ₂₉ : -
		K	IV 18 : z ₃₆ , z ₃₈ : -
		L	IV 21: g, z ₅₁ : -
		U	IV 43 : z ₄ , z ₂₃ : -
			IV 43 : z ₄ , z ₃₂ : -
		V	IV 44 : z ₄ , z ₃₂ : -
		Y	IV 48 : z ₂₉ : -
		Z	IV 50 : z ₄ , z ₂₃ : -
	<i>S. enterica</i> subsp. <i>bongori</i>	R	V 40 : z ₃₅ : -
			V 40 : z ₈₁ : -
		V	V 44 : d: -
			V 44 : z ₃₉ : -
		Y	V 48 : z ₃₅ : -
	<i>S. enterica</i> subsp. <i>indica</i>	S	VI 41 : b: 1,7
		W	VI 45 : a: e,n,x, (z ₁₇)
		Y	VI 48 : z ₁₀ : 1,5
			VI 48 : z ₄₁ : -
			VI 1, v : z ₆₇

Reference source: bgVV Berlin, (Robert von Ostertag Institut, Marienfelde) and Institut für Mikrobiologie und Hygiene, Hamburg (Prof. Aleksic). The strains bearing the

letters TA are strains used for the Ames Test (Maron, D.M. & Ames, B.N., Mutation Research 113, 173-215 (1983)).

Table 1b: Negative strains of non-*Salmonella* species in PCR amplification using the two primer combinations 1 or 2 and the subsequent hybridisation using the oligonucleotide ST 14

No.	Species	Origin
	<i>Bacillus subtilis</i>	ATCC 6051
	<i>Citrobacter freundii</i>	DSM 30040
	<i>Clostridium bifermentans</i>	DSM 630
	<i>Enterobacter agglomerans</i>	IfGB 0202
	<i>Enterobacter cloacae</i>	DSM 30054
	<i>Erwinia carotovora</i>	DSM 30168
	<i>Escherichia coli</i>	ATCC 8739
	<i>Hafnia alvei</i>	IfGB 0101
	<i>Klebsiella oxytoca</i>	DSM 5175
	<i>Klebsiella pneumoniae</i>	ATCC 13883
	<i>Klebsiella oxytoca</i>	DSM 5175
	<i>Lactobacillus spec.</i>	ATCC 20182
	<i>Listeria monocytogenes</i>	ATCC 19118
	<i>Pediococcus domnatus</i>	IfGB 0101
	<i>Proteus vulgaris</i>	DSM 2041
	<i>Pseudomonas fluorescens</i>	DSM 6290
	<i>Serratia marcescens</i>	IfGB 0101
	<i>Shigella flexneri</i>	DSM 4782
	<i>Staphylococcus aureus</i>	ATCC 6538
	<i>Yersinia enterocolitica</i>	DSM 4780

Patent claims 1 to 21 according to Article 34 PCT

1. Set of nucleic acid molecules by means of which, in a process for the detection of representatives of *Salmonella enterica* subsp. *enterica*, *salamae*, *arizonae*, *diarizonae*, *houtenae*, *bongori* and *indica*, all the representatives of those subspecies can be detected, which set of nucleic acid molecules is **obtainable** by

(a) obtaining or deriving a first nucleic acid molecule (nucleic acid molecule 1) in a manner known *per se* using a nucleic acid isolate of a representative of one of the mentioned *Salmonella enterica* subspecies, which first nucleic acid molecule is specifically suitable as primer or probe for the detection of that representative or of further or all representatives of that one *Salmonella enterica* subspecies and possibly also of representatives of further *Salmonella enterica* subspecies,

(b) obtaining or deriving a second nucleic acid molecule (nucleic acid molecule 2) in a manner known *per se* using a nucleic acid isolate of a different representative of one of the mentioned *Salmonella enterica* subspecies, which second nucleic acid molecule is specifically suitable as primer or probe for the detection of that representative or of further or all representatives of that different *Salmonella enterica* subspecies and possibly also of representatives of others of the mentioned *Salmonella enterica* subspecies, and

(c) unless it is already possible to detect all the representatives of the mentioned *Salmonella enterica* subspecies using the nucleic acid molecules obtainable or

derivable according to (a) and (b), continuing to obtain or derive nucleic acid molecules according to (a) and/or (b) until all the representatives of the mentioned *Salmonella enterica* subspecies can be detected using the obtained or derived set of nucleic acid molecules, wherein

(d) the nucleic acid isolates comprise or are phylogenetically conserved base sequences or regions of those base sequences, wherein

(e) the set of nucleic acid molecules starting from the nucleic acid molecules that are obtainable or derivable according to steps (a) to (d) were produced synthetically and in at least two separate synthesis batches, wherein

(f) the individual nucleic acid molecules or some of the nucleic acid molecules hybridise to

(i) different phylogenetically conserved base sequences, or

(ii) one and the same phylogenetically conserved base sequence at non-overlapping sequence regions, or

(iii) one and the same phylogenetically conserved base sequence at overlapping sequence regions,

and wherein

(g) the set for an individual nucleic acid molecule, for a number of its individual nucleic acid molecules or for each of its individual nucleic acid molecules in each case comprises at least one further nucleic acid molecule that, in a region of at least 10 successive nucleotides of their nucleotide chains, corresponds to less than 100% but to at least 80% of the base sequence.

2. Set of nucleic acid molecules by means of which, in a process for the detection of representatives of *Salmonella enterica* subsp. *enterica*, *salamae*, *arizonae*, *diarizonae*, *houtenae*, *bongori* and *indica*, all the representatives of those subspecies can be detected, which set of nucleic acid molecules is **obtainable** by

(a) obtaining or deriving a first nucleic acid molecule (nucleic acid molecule 1) in a manner known *per se* using a nucleic acid isolate of a representative of one of the mentioned *Salmonella enterica* subspecies, which first nucleic acid molecule is specifically suitable as primer or probe for the detection of that representative or of further or all representatives of that one *Salmonella enterica* subspecies and possibly also of representatives of further *Salmonella enterica* subspecies,

(b) obtaining or deriving a second nucleic acid molecule (nucleic acid molecule 2) in a manner known *per se* using a nucleic acid isolate of a different representative of one of the mentioned *Salmonella enterica* subspecies, which second nucleic acid molecule is specifically suitable as primer or probe for the detection of that representative or of further or all representatives of that different *Salmonella enterica* subspecies and possibly also of representatives of others of the mentioned *Salmonella enterica* subspecies, and

(c) unless it is already possible to detect all the representatives of the mentioned *Salmonella enterica* subspecies using the nucleic acid molecules obtainable or derivable according to (a) and (b), continuing to obtain or derive nucleic acid molecules according to (a) and/or (b) until all the representatives of the mentioned *Salmonella*

enterica subspecies can be detected using the obtained or derived set of nucleic acid molecules,

wherein

(d) the nucleic acid isolates comprise or are phylogenetically conserved base sequences or regions of those base sequences,

wherein

(e) the individual nucleic acid molecules or some of the nucleic acid molecules hybridise to

(i) different phylogenetically conserved base sequences,
or

(ii) one and the same phylogenetically conserved base sequence at non-overlapping sequence regions, or

(iii) one and the same phylogenetically conserved base sequence at overlapping sequence regions,

wherein

(f) the set for an individual nucleic acid molecule, for a number of its individual nucleic acid molecules or for each of its individual nucleic acid molecules in each case comprises at least one further nucleic acid molecule that, in a region of at least 10 successive nucleotides of their nucleotide chains, corresponds to less than 100% but to at least 80% of the base sequence,

and wherein

(g) the set of nucleic acid molecules does not comprise any degenerate nucleic acid molecules.

3. Set of nucleic acid molecules by means of which, in a process for the detection of representatives of *Salmonella enterica* subsp. *enterica*, *salamae*, *arizonae*, *diarizonae*, *houtenae*, *bongori* and *indica*, all the representatives of

those subspecies can be detected, which set of nucleic acid molecules is **obtainable** by

(a) obtaining or deriving a first nucleic acid molecule (nucleic acid molecule 1) in a manner known *per se* using a nucleic acid isolate of a representative of one of the mentioned *Salmonella enterica* subspecies, which first nucleic acid molecule is specifically suitable as primer or probe for the detection of that representative or of further or all representatives of that one *Salmonella enterica* subspecies and possibly also of representatives of further *Salmonella enterica* subspecies,

(b) obtaining or deriving a second nucleic acid molecule (nucleic acid molecule 2) in a manner known *per se* using a nucleic acid isolate of a different representative of one of the mentioned *Salmonella enterica* subspecies, which second nucleic acid molecule is specifically suitable as primer or probe for the detection of that representative or of further or all representatives of that different *Salmonella enterica* subspecies and possibly also of representatives of others of the mentioned *Salmonella enterica* subspecies, and

(c) unless it is already possible to detect all the representatives of the mentioned *Salmonella enterica* subspecies using the nucleic acid molecules obtainable or derivable according to (a) and (b), continuing to obtain or derive nucleic acid molecules according to (a) and/or (b) until all the representatives of the mentioned *Salmonella enterica* subspecies can be detected using the obtained or derived set of nucleic acid molecules, wherein

(d) the nucleic acid isolates comprise or are phylogenetically conserved base sequences or regions of those base sequences,

wherein

(e) the individual nucleic acid molecules or some of the nucleic acid molecules hybridise to

(i) different phylogenetically conserved base sequences,
or

(ii) one and the same phylogenetically conserved base sequence at non-overlapping sequence regions, or

(iii) one and the same phylogenetically conserved base sequence at overlapping regions,

wherein

(f) the set for an individual nucleic acid molecule, for a number of its individual nucleic acid molecules or for each of its individual nucleic acid molecules in each case comprises at least one further nucleic acid molecule that, in a region of at least 10 successive nucleotides of their nucleotide chains, corresponds to less than 100% but to at least 80% of the base sequence,

and wherein

(g) the set of nucleic acid molecules comprises only complementing primers.

4. Set according to claim 1, 2 or 3, **characterised** in that the set for an individual nucleic acid molecule, for a number of its individual nucleic acid molecules or for each of its individual nucleic acid molecules in each case comprises at least one further nucleic acid molecule that, in a region of at least 10 successive nucleotides of their nucleotide chains, differs from the other or further nucleic acid molecule in precisely one base position.

5. Set according to any one of the preceding claims, **characterised** in that it comprises one or more, but not exclusively, nucleic acid molecules that are fragments of the SEQ ID NO 1 according to WO 95/00 664 or of its complementary sequence.

6. Set according to any one of the preceding claims, **characterised** in that the individual nucleic acid molecules hybridise to the same strand of nucleic acid isolates of representatives of *Salmonella enterica* subspecies that are being subjected to the process for their detection.

7. Nucleic acid molecule that belongs to a set according to any one of the preceding claims or that can be used for such a set, **characterised** in that, in a region of at least 10 successive nucleotides of its nucleotide chain, the sequence of the nucleic acid molecule corresponds exactly to a sequence region of at least one representative of *Salmonella enterica* subspecies according to any one of claims 1, 2 or 3, the sequence region comprising or being a phylogenetically conserved base sequence or a region of that base sequence.

8. Nucleic acid molecule according to claim 7, **characterised** in that, in a region of at least 10 successive nucleotides of its nucleotide chain, it is 100% or at least 80% identical to a corresponding number of successive nucleotides of one or more of the following sequences or their complementary sequences:

SEQ ID NO: 1 ATGGATCAGAATACGCCCCG

SEQ ID NO: 2 ATGGATCAGAATACACCCCG

SEQ ID NO: 3 CAGAATACGCCCCGTTTCGGC
SEQ ID NO: 4 CAGAATACACCCCGTTTCGGC
SEQ ID NO: 5 CAGAATACGCCCCGTTTCAGC
SEQ ID NO: 6 CAACCTAACTTCTGCGCCAG
SEQ ID NO: 7 CAACCTAACTTCTGCACCAG
SEQ ID NO: 8 CAACCTAACCTCTGCGCCAG
SEQ ID NO: 9 CAACCTAACTTCTGCGGCAG
SEQ ID NO: 10 CAGCCTAACTTCTGCGCCAG

9. Nucleic acid molecule **characterised** in that, in respect of its sequence, it is homologous to a nucleic acid molecule according to either claim 7 or claim 8 and, in at least 10 successive nucleotides of its nucleotide chain,
(i) is identical to a nucleic acid molecule according to either claim 7 or claim 8, or
(ii) differs from a nucleic acid molecule according to either claim 7 or claim 8 in not more than one nucleotide, or
(iii) differs from a nucleic acid molecule according to either claim 7 or claim 8 in not more than two nucleotides.

10. Nucleic acid molecule according to any one of claims 7 to 9, **characterised** in that it is from 10 to 250 nucleotides long, and preferably from 15 to 30 nucleotides long.

11. Nucleic acid molecule according to any one of claims 7 to 10, **characterised** in that it is single-stranded or has a complementary strand.

12. Nucleic acid molecule according to any one of claims 7 to 11, **characterised** in that it is present

- (i) as DNA, or
- (ii) as RNA corresponding to (i), or
- (iii) as PNA, the nucleic acid molecule where appropriate having been modified or labelled in a manner known *per se* for analytical detection processes, especially detection processes based on hybridisation and/or amplification.

13. Nucleic acid molecule according to claim 12, **characterised** in that it is a modified or labelled nucleic acid molecule in which up to 20% of the nucleotides of at least 10 successive nucleotides of its nucleotide chain are building blocks known *per se* as probes and/or primers, especially nucleotides that do not occur naturally in bacteria.

14. Nucleic acid molecule according to claim 12 or claim 13, **characterised** in that it is a modified or labelled or additionally modified or labelled nucleic acid molecule that comprises, in a manner known *per se* for analytical detection processes, one or more radioactive groups, coloured groups, fluorescent groups, groups for immobilisation on a solid phase, groups for an indirect or direct reaction, especially for an enzymatic reaction, preferably using antibodies, antigens, enzymes and/or substances having an affinity for enzymes or enzyme complexes, and/or other modifying or modified groups of nucleic-acid-like structure that are known *per se*.

15. Kit for analytical detection processes, for the detection of bacteria of the *Salmonella* genus, **characterised** by

- (i) a set of nucleic acid molecules according to any one of claims 1 to 6, or
- (ii) one or more nucleic acid molecules according to any one of claims 7 to 14.

16. Kit according to claim 15, **characterised** in that the set of nucleic acid molecules was produced synthetically and that it was produced in at least two separate synthesis batches.

17. Kit according to claim 16, **characterised** in that the kit does not comprise any degenerate nucleic acid molecules.

18. Use of a set of nucleic acid molecules according to any one of claims 1 to 6, of one or more nucleic acid molecules according to any one of claims 7 to 14 or of a kit according to any one of claims 15 to 17 to detect the presence or absence of bacteria belonging to representatives of *Salmonella enterica* subspecies according to claim 1, 2 or 3.

19. Use according to claim 18, **characterised** in that nucleic acid hybridisation and/or nucleic acid amplification is carried out.

20. Use according to claim 19, **characterised** in that a polymerase chain reaction (PCR) is carried out as nucleic acid amplification.

21. Use according to claim 18, 19 or 20, **characterised** in that differences between the genomic DNA and/or RNA of the bacteria to be detected and of the bacteria that are not to be detected are determined at at least one nucleotide position in the region of a nucleic acid molecule according to any one of claims 7 to 14 and representatives of a group of bacteria of the *Salmonella* genus are detected, especially representatives of *Salmonella enterica* subspecies according to claim 1, 2 or 3.

Abstract

The present invention relates to a nucleic acid molecule or molecules and to a process for the detection of bacteria of the *Salmonella* genus. The invention relates also to a test kit or test kits for carrying out the mentioned detection processes.

continued on next page

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PTO/SB01 (2-15)

OMB 051-0032

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Attorney Docket Number 2727-100J

First Named Inventor Cornelia Berghof

COMPLETE IF KNOWN

Application Number 09/485,434

Filing Date

Group Art Unit

Examiner Name

DECLARATION FOR
UTILITY OR DESIGN
PATENT APPLICATION☐ Declaration
Submitted
with Initial Filing☒ Declaration
Submitted after
Initial Filing

As a duly named inventor, I hereby declare that:

My residence, past office address, and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention created:

"Nucleic Acid Molecule Set for Detecting Salmonella,
Nucleic Acids, Kit and Use"

(Title of the Invention)

The identification of which

☐ is claimed herein
OR☒ was filed on (MM/DD/YYYY) 08/12/1998

as United States Application Number or PCT International

(USSN 09/485,434)

Application Number PCT/EP98/05129

and was amended on (MM/DD/YYYY)

(if applicable)

I hereby state that I have reviewed and understood the contents of the above identified specification, including the claims, as amended by any amendments specifically referred to above.

I acknowledge the duty to disclose information which is material to patentability as defined in Title 37 Code of Federal Regulations, §1.56

I hereby claim through priority benefits under Title 35, United States Code §119 (a)-(d) or §365(a) of any foreign application(s) for patent by inventor's certificate, or §362 (a) of any PCT international application which designated at least one country other than the United States of America, filed before and have also indicated below, by checking the box, any foreign application for patent or inventor's certificate, or at any PCT international application having a filing date before that of the application on which priority is claimed.

Prior Foreign Application (Number)	Country	Foreign Filing Date (MM/DD/YYYY)	Priority Not Claimed	Certified Copy Attached?	
				YES	NO
197 34 940.4	Germany	08/12/1997	<input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/>	<input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/>	<input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/>
<input type="checkbox"/> Additional foreign application numbers are listed on a supplemental priority sheet attached hereto.					
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Application Number(s)	Filing Date (MM/DD/YYYY)	<input type="checkbox"/> Additional foreign application numbers are listed on a supplemental priority sheet attached hereto.			

Sworn Under Oath: This form is executed in triplicate and returned to the inventor. This will vary depending upon the needs of the individual case. Any changes or the amount of time you are required to complete this form should be sent to the Chief Information Officer, Patent and Trademark Office, Washington, DC 20231. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Assistant Commissioner for Patents, Washington, DC 20231.

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DECLARATION				Page 2	
<p>I hereby declare the applicant under Title 35, United States Code §120 of any United States patent, or §364(a) of any PCT international publication designating the United States of America, legal holder and, together with the INVENTOR, holder of the claims of this application to be disclosed in the prior United States or PCT international publication in the manner provided by the first paragraph of Title 35, United States Code §122. I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations §1.56 which becomes available between the first day of the first publication and the day of the PCT international publication from date of the publication.</p>					
U.S. Parent Application Number	PCT Parent Number	Parent Filing Date (MM/DD/YYYY)	Parent Patent Number (if applicable)		
<input type="checkbox"/> Additional U.S. or PCT international publication numbers are listed on a supplemental sheet attached hereto.					
<p>As a related inventor, I hereby declare the following attorney(s) and/or agent(s) to be authorized to prosecute this application and to bind me as inventor in the future and to execute any necessary papers:</p>					
<input type="checkbox"/> Firm Name <input type="checkbox"/> OR <input type="checkbox"/> Last attorney(s) and/or agent(s) name and registration number below		Customer or Agent <input type="checkbox"/>			
Name	Registration Number	Name	Registration Number		
Joseph C. Sullivan	18,720	Ronald R. Santucci	28,988		
Gerald Levy	24,419	Ronald E. Brown	32,200		
		John F. Gulbin	33,180		
		Richard J. Danyko	33,672		
<input type="checkbox"/> Additional attorney(s) and/or agent(s) named on a supplemental sheet attached hereto.					
Please send all correspondence to: <input type="checkbox"/> Customer or Agent Number		OR <input type="checkbox"/> Fill in correspondence address below			
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Address	Pitney, Hardin, Ripp & Gueh, LLP				
Address	711 Third Avenue, 20th Floor				
City	New York,	State	NY		ZIP 10017
Country	U.S.A.	Telephone	212-687-6000		Fax 212-682-3484
<p>I hereby declare that all statements made herein or by any agent or attorney are true and that all statements made on information and belief are believed to be true, and further that these statements were made with the knowledge that such false statements and the lies so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may constitute the crime of the falsification of any document created through</p>					
Name of Sole or First Inventor:		<input type="checkbox"/> A person has been filed for this unnamed inventor			
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Inventor's Signature	<i>Ze flis</i>		Date	7.4.2000	
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(Declaration for Utility or Design Patent Application (PTO/SB/01) [1-10.1]—page 2 of 3)

06.: ANALYTICON AG;

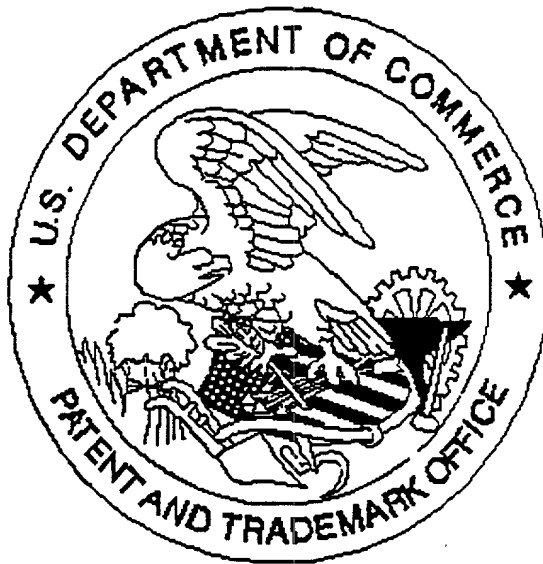
+49 331 2300100;

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Seite 5/5

DECLARATION				ADDITIONAL INVENTOR(S) Supplemental Sheet			
Name of Additional Joint Inventor, if any:				<input type="checkbox"/> A petition has been filed for this unsigned inventor			
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Given Name	<u>Pia</u>	Middle Initial		Family Name	<u>Scheu</u>	Suffix	
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Inventor's Signature	<u>Freimut Wilborn</u>			Date	<u>April 6, 2000</u>		
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City	<u>Berlin</u>	State		Zip	<u>D-14057</u>	Country	<u>Germany</u>
Name of Additional Joint Inventor, if any:				<input type="checkbox"/> A petition has been filed for this unsigned inventor			
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